

Prevention of Benzene-Induced Myelotoxicity by Nonsteroidal Anti-Inflammatory Drugs

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Benzene affects hematopoietic progenitor cells leading to bone marrow depression and genotoxic effects such as micronucleus formation. Progenitor cell proliferation and differentiation are inhibited by prostaglandins produced by macrophages. Administration of benzene to DBA/2 or C57BL/6 mice caused a dose-dependent bone marrow depression and a significant increase in marrow prostaglandin E level and both were prevented by the coadministration of indomethacin and other inhibitors of the cyclooxygenase component of prostaglandin H synthase. Levels of benzene that decreased bone marrow cellularity also caused genotoxic effects measured as increased micronucleated polychromatic erythrocytes in peripheral blood, which was also prevented by the coadministration of indomethacin. These results suggest a possible role for prostaglandin synthase in benzene myelotoxicity; a mechanism by which this might occur is presented.

Introduction

Chronic exposure of humans and experimental animals to benzene results in bone marrow depression, which can lead to pancytopenia and aplastic anemia (1). Benzene exposure also results in genotoxic effects such as structural chromosome aberrations (2) and single-strand breaks that produce a prolonged accumulation of micronuclei in mature erythrocytes of the peripheral blood in rodents (3-9). Benzene is also associated with an increased incidence of acute myelogenous leukemia and some of its variant forms in humans and several types of solid tumors and leukemia and lymphomas in rats and mice (10).

Benzene metabolism, which is required for toxicity, occurs predominantly in the liver where phenol, the major metabolite, is converted to the secondary metabolites hydroquinone and catechol (11,12). Phenol, transported from the liver to the bone marrow, is metabolized by a peroxidase-mediated pathway as is its oxidation product, hydroquinone (13,14), which can be converted to *p*-benzoquinone via the benzosemiquinone radical (15).

In bone marrow, benzene appears to have a cytotoxic effect on hematopoietic progenitor cells in intermediate

stages of differentiation regardless of cell lineage (16,17), but there is also evidence to indicate that injury to marrow stromal cells may be an important factor in benzene-induced myelosuppression (18,19). These effects may be related since hematopoiesis results from stem and progenitor cells interacting with the supporting marrow stroma. Stromal cells provide essential growth factors [colony-stimulating factors (CSFs)] (20) and a favorable microenvironment for the survival and the regulated proliferation and differentiation of stem and progenitor cells (21).

Macrophages in bone marrow stroma have been suggested to be an important regulator of hematopoiesis (22). Hematopoiesis is enhanced when the macrophage releases the monokine IL-1 (23), which induces stromal cells to produce CSFs (24,25), progenitor cell growth factors. Downregulation of hematopoiesis occurs when CSFs induce macrophages (26-28) and fibroblasts (29) to produce prostaglandins. Prostaglandin E₂ blocks the ability of myeloid progenitor cells to respond to CSFs (30) and inhibits the production of IL-1 in the macrophage (31). The macrophage can thereby regulate hematopoiesis by producing CSFs to enhance hematopoietic proliferation and downregulate it by increasing the concentration of prostaglandins. Consequently, an effect of benzene or its metabolites on stromal macrophages could secondarily affect the survival, proliferation, and differentiation of progenitor cells (21).

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Garnett et al. (32) reported that the development of an adherent stromal layer in culture from marrow cells of benzene-treated animals was morphologically altered, and its ability to support the differentiation of stem cells seeded upon it was decreased, thus demonstrating an *in vivo* effect of benzene on stromal cells. Exposure of bone marrow stromal cells *in vitro* to phenol, hydroquinone, or catechol causes similar effects on the stromal cells (33). Gaido and Wierda (19) reported that hydroquinone increased production of PGE₂ in marrow stromal cell cultures to a level that significantly inhibited stromal cell-supported granulocyte/macrophage (G/M) colony formation. Pretreatment of the cultures with the nonsteroidal anti-inflammatory drug (NSAID) indomethacin, an inhibitor of the cyclooxygenase component of prostaglandin synthase, decreased PGE₂ levels and protected against hydroquinone toxicity (19,34).

These important results suggest a possible role for prostaglandin synthase in benzene-induced myelo- and genotoxicity. Since prostaglandins are inhibitors of hematopoiesis, the stimulation of their formation by benzene could cause an inhibition of stromal cell-supported hematopoiesis. Also, the endoperoxidase component of prostaglandin synthase could oxidize phenol and/or hydroquinone to reactive metabolites. For example, hydroquinone could be metabolized to *p*-benzoquinone, which induces single-strand breaks in DNA (35) and micronucleus formation in polychromatic erythrocytes (36). We report here that the depression of bone marrow cellularity and the increase in frequency of micronucleated polychromatic erythrocytes induced in mice by benzene can be prevented by the coadministration of NSAID.

Materials and Methods

Materials

Animals. Swiss-Webster wild-type male mice were obtained from Ace Breeders (Boyertown, PA); DBA/2 and C57BL/6 inbred male mice were purchased from Jackson Laboratories (Bar Harbor, ME). The animals weighed between 15 and 25 g when used. Animals were housed in an AAALAC-approved central animal facility and fed Purina Rodent Chow *ad libitum*.

Reagents. Benzene (spectroanalyzed and thiophene-free) was purchased from Fisher Scientific Company (Pittsburgh, PA). Indomethacin, fluorescein diacetate, ethidium bromide, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), dextran (70,000 m.w.), activated charcoal (250–350 mesh), and bovine serum albumin (BSA) were obtained from the Sigma Chemical Company (St. Louis, MO). Roswell Park Memorial Institute (RPMI) 1640 medium and trypan blue dye were purchased from Hazelton Dutchland (Denver, PA). Meclofenamate was a gift from John Flynn of Thomas Jefferson University. Heparin was obtained from the Nutritional Biochemical Corporation (Cleveland, OH) and aspirin was purchased from Merck and Company (Rahway, NJ). NEN/Dupont (Boston, MA) was the source of

Aquasol-2 and [³H]PGE₂ (170 Ci/mmol). PGE₂ was obtained from CalBiochem/Behring (San Diego, CA), and PGE₂ antiserum was from Advanced Magnetics, Inc. (Cambridge, MA). Colchicine was a gift from James Kocsis of Thomas Jefferson University. All chemicals were of the highest grade available.

Methods

Handling of Benzene. The laboratory practices recommended by the NIH Guidelines for the Laboratory Use of Chemical Carcinogens were followed. During the period when the animals were expiring benzene, they were housed in a carcinogen-rated hood under conditions approved by the Institutional Animal Research Committee.

Treatment of Animals with Chemicals. Benzene in corn oil (12 mL/kg body weight) was administered IP at doses of 0, 400, 800, or 1200 mg/kg twice daily for 2 days. Daily injections of animals were separated by 7 hr. The animals were sacrificed by cervical dislocation on day 3 and one femur per animal was used. Indomethacin was prepared as a fresh stock solution (2 mg/mL in 95% ethanol) and diluted with Dulbecco's phosphate-buffered saline without calcium or magnesium, pH 7.3 (PBS A), to a final concentration of 0.08 or 0.17 mg/mL. Aspirin (4.15 mg/mL) was prepared in 50 mM sodium bicarbonate prior to each injection and a solution of meclufenamate (0.48 or 0.96 mg/mL) was prepared daily in water. In the experiments with prostaglandin synthase inhibitors, benzene was administered at 400 mg/kg body weight and the inhibitors were coadministered at doses described. Control animals received corn oil and/or a 4.2% solution of ethanolic PBS A.

Determination of Bone Marrow Cellularity. The epiphyseal plate on each end of the femur was removed. Three milliliters of a solution of cold RPMI 1640 medium supplemented with 10 U/mL heparin and 10 mM HEPES, pH 7.3, was forced through the femur using a syringe with a 25-gauge needle. The bone marrow cells were flushed into a tissue culture tube and collected by centrifugation at 1000g at 4°C for 5 min. Red blood cells were used by suspending the marrow cells in 1 mL of a cold solution consisting of 155 mM NH₄Cl, 0.1 mM EDTA, and 10 mM KHCO₃. The nucleated cells were collected by centrifugation, and the pellet was dispersed into a single cell suspension in 5 mL of RPMI 1640 medium supplemented with 10 mM HEPES, pH 7.3, that was placed on ice for 2 min to allow red cell membranes to sediment. The nucleated cells were collected by centrifugation, suspended in RPMI 1640-HEPES, counted with the use of a hemocytometer, and tested for viability using both the trypan blue dye and fluorescein diacetate/ethidium bromide methods (37).

Determination of Micronuclei. Immediately after sacrifice, a blood sample was obtained and a smear made to evaluate clastogenicity from benzene in the form of increased frequency of micronucleus formation in polychromatic erythrocytes (PCE) of peripheral blood. In general, the assay was carried out under the guidelines published

for the conduct of micronucleus assays (38). The blood was smeared on the slide in a thin uniform layer that allowed for the visualization of individual cells. Blood cells were fixed in 100% methanol for 15 min and allowed to dry overnight. The smears were stained for 6 min in a solution of acridine orange (0.125 mg/mL) in phosphate buffer, pH 7.4 (39), after which they were washed in phosphate buffer for an additional 7 min. Fluorescent microscopy was used to distinguish normochromatic erythrocytes (NCE) from PCE. In each animal, the frequency of micronucleated cells was determined among 2000 NCE and 500 PCE; concomitantly the number of PCE/2000 NCE was also evaluated. The slides were randomly coded. Structures refractile when in focus were not scored.

Effect of Benzene on PGE₂ Levels. DBA/2 mice were injected with benzene (800 mg/kg in corn oil) in the presence or absence of indomethacin (8 mg/kg). Control animals received equal volumes of vehicles. The animals were killed 6 to 7 hr after benzene administration, both femurs were dissected out, and the epiphyseal plate on each end was removed. Each femur was flushed with 0.1 ml of ice-cold 10 mM phosphate buffer, pH 7.0, containing 150 mM NaCl, 0.1% BSA and 20 µg/mL meclofenamate (RIA buffer). Cells were combined, suspended, and collected by centrifugation at 500g for 10 min at 4°C. Marrow cells were suspended in 1 mL of cold lysing solution, centrifuged, and washed three times with 1 mL PBS A. The protein content of the nucleated cells was determined by the method of Bradford (40) using bovine serum albumin as a standard.

Determination of PGE. The marrow supernatant was analyzed for total PGE by radioimmunoassay according to the procedure supplied by Advanced Magnetics (Cambridge, MA). Incubations contained 0.1 mL of PGE₂ antiserum (1:5000), [³H]PGE₂ (38000 dpm), and PGE₂ (0.04 to 15 ng or an amount of marrow supernatant) to a final volume of 0.3 mL. Cross-reactivity of the PGE₂ antiserum was 100% with PGE₁; 6.0% with PGA₂; and 3.0% with PGA₁. Radioactivity bound to the antibody was determined in a 0.5 mL sample added to 5.0 mL of Aquasol-2. Radioactivity was counted in an intertechnique SL 30 scintillation counter.

Preparation of Macrophages. Peritoneal macrophages were elicited from male C57BL/6 mice 5 days following protease peptone (IP). Macrophages were purified by active adherence to plastic Petri dishes. Over 95% of adherent cells are macrophages as assessed by morphology, nonspecific esterase-positive staining, and phagocytosis of sheep red blood cells.

Statistical Analyses. One-way ANOVA (41) followed by Dunnett's *t*-test (42) was used to analyze bone marrow cellularity and PGE levels. The PCE/NCE were analyzed by one-way ANOVA (41), and if the ratios were not significantly different a one-way ANOVA of the micronuclei/PCE was carried out. If this proved to be significant, a Duncan's multiple range test was carried out (43). Results are expressed as mean values ± standard deviation (SD). A value of $p \leq 0.05$ was considered to be significant.

Results

Depression of Bone Marrow Cellularity as a Function of Benzene Dose

The dose of benzene that gave sufficient depression of the bone marrow cellularity without killing the animals was previously determined (44). The degree of bone marrow depression was a function of benzene dose in both DBA/2 and C57BL/6 strains of mice over a concentration range of 200 to 1200 mg/kg body weight. A dose of 400 mg/kg body weight caused a statistically significant ($p \leq 0.005$) decrease in marrow cellularity in both strains of mice with no loss of animals and with greater than 95% viability of the cells flushed from the femur. This dose of benzene was generally used in most subsequent experiments.

Prevention of Benzene-Induced Bone Marrow Depression by NSAID

Treatment of DBA/2 mice with benzene twice daily for 2 days caused a significant decrease in bone marrow cellularity when measured on day 3 (Table 1). Coadministration of indomethacin (2 mg/kg body weight) with benzene completely prevented the marrow depression. This experiment was repeated five times with the same results. Similar results were obtained using C57BL/6 mice (data not shown). The ability of indomethacin to prevent the benzene-induced bone marrow depression was dose related and indomethacin *per se* had no effect on bone marrow cellularity (Table 1).

Meclofenamate and aspirin, which inhibit the cyclooxygenase activity of prostaglandin H synthase by mechanisms different from that of indomethacin, were tested for their ability to prevent benzene toxicity. These results, which are presented in Table 1, indicate that meclofenamate (4 mg/kg) or aspirin (50 mg/kg) significantly prevented benzene-induced bone marrow depression. The agents by themselves had no effect on bone marrow cellularity.

Effect of Benzene on PGE Levels in Bone Marrow

Total PGE in the bone marrow of animals treated with benzene was significantly higher than that present in the marrow of animals receiving only the vehicle when PGE was expressed per femur or per milligram cellular protein (Table 2). Coadministration of indomethacin with benzene not only prevented the increase in PGE due to benzene, but also significantly lowered the amount of PGE normally produced in marrow (Table 2).

Benzene-Induced Micronucleus Formation

Under conditions where benzene causes significant myelotoxicity in the form of decreased bone marrow cellularity (Table 1), it also causes genotoxicity measured as an increase in the frequency of micronucleus formation in PCE. It can be seen in Table 3 that benzene administered to Swiss-Webster mice at 400 mg/kg body weight caused

Table 1. Prevention of benzene-induced bone marrow depression by prostaglandin H synthase inhibitors.

Group	Nucleated bone marrow cells, $\times 10^7$ /femur	% of control
Experiment 1 ^{a,b}		
Control ^c	1.44 \pm 0.08	100
+ Benzene ^d	0.91 \pm 0.07*	63
+ Benzene + indomethacin ^e	1.43 \pm 0.06	99
+ Indomethacin	1.48 \pm 0.07	102
Experiment 2 ^{a,b}		
Control ^c	1.05 \pm 0.07	100
+ Benzene ^d	0.62 \pm 0.16*	60
+ Benzene + meclofenamate ^f	0.96 \pm 0.09	91
+ Meclofenamate	1.03 \pm 0.13	100
Experiment 3 ^{a,b}		
Control ^c	1.62 \pm 0.14	100
+ Benzene ^d	0.97 \pm 0.12†	60
+ Benzene + aspirin ^g	1.46 \pm 0.19	90
+ Aspirin	1.55 \pm 0.19	96

^aDBA/2 mice were used in experiments 1 and 2, and C57BL/6 mice were used in experiment 3.

^bAnimals received treatment twice daily for 2 days and on day 3 bone marrow cellularity was determined. Values are reported as \pm SD of $n = 3$ in experiments 1 and 2 and $n = 4$ in experiment 3.

^cControl animals received corn oil and water or 50 mM sodium bicarbonate.

^dBenzene was administered IP at 400 mg/kg.

^eIndomethacin was administered IP at 2 mg/kg.

^fMeclofenamate was administered IP at 4 mg/kg.

^gAspirin was administered IP at 50 mg/kg.

* $p \leq 0.01$ compared to the control.

† $p \leq 0.001$ compared to the control.

a 3.8-fold increase in the frequency of micronucleus formation in PCE under conditions where the same dose caused approximately a 40% decrease in bone marrow cellularity (Table 1). The number of micronuclei observed in the control group agrees with the spontaneous frequency of micronucleus formation in PCEs reported for mice (45). Colchicine, an alkaloid known to cause micronuclei by interfering with spindle formation and thus with nuclear division in bone marrow erythroblasts, was used as a positive control (approximately 15 micronuclei/1000 PCE). A change in the frequency of micronuclei in NCE was not observed and would not be expected since the frequency of micronucleated cells among NCE does not increase as markedly as that among PCEs after an acute exposure (38). The lack of an increase in micronuclei in NCE also serves as a quality control since any artifacts in a given smear will produce apparent increases in the frequencies of micronuclei in both NCE and PCE (38).

Table 2. Modulation of prostaglandin E levels in bone marrow by benzene and indomethacin.

Group	Bone marrow PGE	
	pg/femur	pg/mg protein
Control ^a	29.8 \pm 8.32	371 \pm 120
+ Benzene ^b	46.4 \pm 11.8*	574 \pm 200*
+ Benzene + indomethacin ^c	14.3 \pm 4.28*	191 \pm 65*

^aMarrow was removed from both femurs of DBA/2 mice and analyzed for PGE and protein as described in "Methods." Values represent the means \pm SD; $n = 6$ –8 mice per group.

^bBenzene-treated animals received 800 mg/kg, IP.

^cIndomethacin-treated animals received 8 mg/kg, IP, in addition to the benzene.

* $p \leq 0.01$ compared to controls.

Prevention of Benzene-Induced Genotoxicity by Indomethacin

We carried out experiments to ascertain whether NSAID could also prevent the genotoxic effects of benzene. As can be seen for the data presented in Table 3, coadministration of indomethacin with benzene completely prevented the increase in the frequency of micronucleated PCE without affecting the division or maturation of nucleated erythroid cells as indicated by the PCE/NCE. The administration of indomethacin alone had no effect on the frequency of micronucleus formation. This experiment was repeated six times with essentially the same result.

Arachidonic Acid-Dependent Activation of Phenol by Macrophages

The inhibition of the genotoxic effects of benzene by indomethacin suggested that metabolites of benzene such as phenol and/or hydroquinone might be converted to clastogenic compounds by prostaglandin synthase endoperoxidase. Consequently, we tested the ability of macrophages to effect the arachidonic acid-dependent metabolism of phenol to reactive species that bind to macromolecules. Purified peritoneal macrophages were incubated with [¹⁴C] phenol in the presence and absence of arachidonic acid. As can be seen in Figure 1, macrophages metabolize phenol to reactive compounds that irreversibly bind to macromolecules in a reaction that is dependent on the concentration of arachidonic acid in the incubation. These results suggest that prostaglandin syn-

Table 3. Induction of micronuclei by benzene and its prevention by indomethacin.

Group ^a	Micronuclei/10 ³		PCE/NCE
	PCE	NCE	
Experiment 1			
Control ^b	2.8 ± 1.0	2.3 ± 0.9	34.3 ± 3.6
+ Benzene ^c	14.2 ± 1.7*	2.2 ± 0.8	26.9 ± 3.9
+ Benzene + indomethacin ^d	4.5 ± 1.0	2.0 ± 0.8	24.6 ± 5.0
Experiment 2			
Control ^b	4.2 ± 1.3	1.6 ± 0.6	28.8 ± 5.0
+ Benzene ^c	12.2 ± 1.3†	2.0 ± 0.0	31.1 ± 4.5
+ Benzene + indomethacin ^d	5.6 ± 2.6	1.8 ± 0.7	26.7 ± 5.0

^aEach group consisted four Swiss-Webster wild-type male mice. Data are expressed as mean ± SD.

^bControl animals received corn oil and/or a solution of 4.2% ethanolic PBS A.

^cBenzene (400 mg/kg in corn oil) was administered twice daily for 2 days.

^dIndomethacin (2 mg/kg in PBS A) was coadministered IP with benzene.

^eBenzene (1000 mg/kg in corn oil) was administered as in experiment 1.

* $p \leq 0.01$ compared to the control group and benzene + indomethacin-treated group.

† $p \leq 0.001$ compared to the control group and benzene + indomethacin-treated group.

thase endoperoxidase in macrophages might be responsible for the cooxidation of phenol and/or hydroquinone to reactive species during the benzene-induced formation of prostaglandins. Purified prostaglandin H synthase has been shown to oxidize both phenol and hydroquinone to species that bind irreversibly to protein and DNA (46).

Discussion

Benzene caused a dose-dependent depression of bone marrow cellularity in both DBA/2 and C57BL/6 mice, which was prevented by the coadministration of indomethacin, aspirin or meclofenamate (Table 1). Indomethacin protects against benzene-induced bone marrow depression at a dose (2 mg/kg, 2 times/day for 2 days) that has no effect on body weight or bone marrow cellularity. Benzene also causes genotoxicity as measured by an increase in the frequency of micronucleus formation in polychromatic erythrocytes, which also can be prevented by indomethacin (Table 3).

Under the conditions where benzene caused bone marrow depression and increased the frequency of

micronucleated PCE, liver microsomes from indomethacin-treated animals showed no alteration in cytochromes P-450 level (nmole/mg microsomal protein) or activity as measured by either the conversion of benzene to phenol or the *N*-demethylation of aminopyrine (data not presented). This indicates that indomethacin does not interfere with the initial metabolism of benzene to phenol in the liver.

Benzene increased the level of PGE (Table 2) and undoubtedly that of other prostaglandins (PGE_{2α}) in bone marrow and depressed bone marrow cellularity. Coadministration of indomethacin prevented the benzene-induced rise in PGE level (Table 2).

The ability of inhibitors of prostaglandin synthase to prevent the increase in PGE level in bone marrow and concomitantly prevent both benzene-induced bone marrow cell depression and micronucleus formation suggests that prostaglandin synthase may play a role in the expression of benzene-induced toxicity; a possible mechanism is presented in Figure 2. Benzene is metabolized in the liver and bone marrow to phenol and hydroquinone. Benzene *per se* may act on bone marrow cells to effect the constitutive release of arachidonic acid from membrane phospholipids.

Rhogani et al. (47) have recently demonstrated that benzene, albeit at rather high concentrations, can activate purified protein kinase C from brain and also the enzyme in platelets. Activators of kinase C (i.e., phorbol esters) are known to enhance the release of arachidonic acid from phospholipids in platelets (48) and macrophages (49). Alternatively, a highly reactive metabolite such as *p*-benzoquinone might interact with and damage membrane components, causing a turnover of phospholipids and the release of arachidonic acid (50). In either case, released arachidonic acid would be converted by the cyclooxygenase component of prostaglandin synthase to the hydroperoxide (PGG₂). The hydroperoxide in turn would drive the endoperoxidase activity of the enzyme. The cooxidation of hydroquinone or phenol during endoperoxidase conversion of PGG₂ to PGH₂, the immediate precursor molecule for prostaglandins, would result in increased levels of prostaglandins. In the case of hydroquinone,

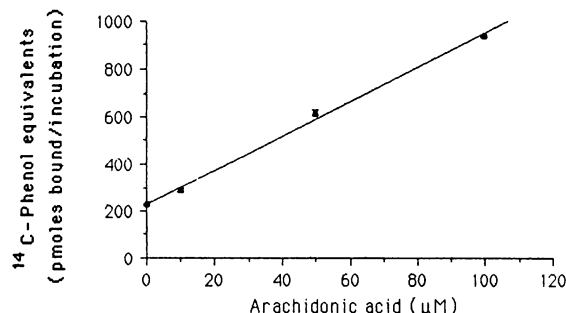


FIGURE 1. Arachidonic acid-dependent activation of phenol by macrophages. Incubation mixtures contained 2.3×10^6 macrophages, 0.25 mM [¹⁴C] phenol (8000 dpm/nmole), 0.1 mM arachidonic acid diluted to a final volume of 1 mL with RPMI-1640 buffered to pH 7.3 with 5 mM HEPES. Reactions were terminated after 30 min by the addition of trichloroacetic acid. Irreversible binding of [¹⁴C] phenol equivalents was assessed by liquid scintillation counting after extensive washes with acetone/hexane/methanol.

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